

# Transcriptional regulation of the heavy subunit chain of $\gamma$ -glutamylcysteine synthetase by ionizing radiation

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**Abstract** Since glutathione (GSH) protects against oxidative stress, we determined the regulation of cellular GSH by ionizing radiation in human hepatoblastoma cells, HepG2. The levels of GSH increased in irradiated HepG2 due to a greater  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) activity, which was paralleled by  $\gamma$ -GCS heavy subunit chain ( $\gamma$ -GCS-HS) mRNA levels. Transcription of deletion constructs of the  $\gamma$ -GCS-HS promoter cloned in a reporter vector was associated with activator protein-1 (AP-1), consistent with the DNA binding of AP-1 in nuclear extracts of irradiated HepG2. Hence, the transcriptional regulation of  $\gamma$ -GCS by ionizing radiation emerges as an adaptive mechanism, which may be of significance to control the consequences of the oxidative stress induced by radiation.

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**Key words:** Oxidative stress; Gene regulation; Glutathione; Transcription factor; Radiobiology; NF- $\kappa$ B; AP-1

## 1. Introduction

One of the best characterized biological effects of ionizing radiation is the overgeneration of ROS [1,2], which interacts with cellular macromolecules and participates in the chain of events that culminate in cell death. To control these potential harmful effects of ROS overproduction, irradiated cells may trigger adaptive mechanisms, such as induction of cellular antioxidants [3]. Indeed, previous studies have demonstrated that overexpression of Mn-SOD affords survival of tumor cells to ionizing radiation, highlighting the relevant role of antioxidants in the control of the oxidative stress induced by radiation [3].

Glutathione (GSH), the main nonprotein cellular thiol, plays a prominent role in the defense against oxidative stress-induced cell injury [4–6]. To fulfil such a role, reduced GSH acts as substrate for the GSH S-transferase and GSH peroxidase, downplaying the levels of ROS and of a wide variety of toxic compounds including many chemotherapeutic and alkylating drugs [7–9]. Conjugation of these agents with GSH generally results in their inactivation and, in some cases, facilitates their excretion from cells. Furthermore, due to its

protective role in maintaining critical cellular functions, certain cancer cells develop resistance against chemotherapeutic agents by up-regulating the GSH levels [10,11].

GSH is synthesized from its constituent amino acids in two sequential enzymatic reactions, catalyzed by  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) and GSH synthetase.  $\gamma$ -GCS catalyzes the rate limiting step in de novo GSH synthesis, and is inhibited by GSH through a feedback mechanism.  $\gamma$ -GCS has been shown to be up-regulated in multiple cell types in response to a wide variety of stimuli, including alkylating compounds, chemotherapeutic agents and cigarette smoke [12–17]. In this regard, although previous studies have shown that certain strains of *E. coli* endowed with a greater capacity to synthesize GSH by gene transfer are resistant to radiation [18], the regulation of  $\gamma$ -GCS in response to radiation has not been reported to the best of our knowledge. Here, we provide evidence that ionizing radiation increases cellular GSH level by transcriptional regulation of the heavy chain of  $\gamma$ -GCS, which may constitute an adaptive response to control the consequences of ROS and the oxidative stress induced by radiation.

## 2. Materials and methods

GSH, GSSG, cysteine and BSO were obtained from Sigma Chemical Co. (St. Louis, MO). Monochlorobimane and DCFDA were from Molecular Probes (Eugene, OR). Trizol LS reagent and lipofectamine were obtained from Life Technologies (Gaithersburg, MD).  $\gamma$ -Glutamylcysteine was prepared by enzymatic hydrolysis of GSSG and reduction with dithiothreitol prior to use as described [19]. The human hepatoblastoma cell line, HepG2, was purchased from ATTC and routinely cultured in DMEM containing 10% FBS, penicillin and streptomycin (0.1 mg/ml) in a humidified atmosphere of 5% CO<sub>2</sub>/95% air, at 37°C and were subcultured every 7 days, changing culture medium every 3–4 days.

### 2.1. Irradiation of HepG2

Subconfluent HepG2 cells were pelleted and washed once with PBS and resuspended at  $10 \times 10^6$  cells/ml. To ensure a homogeneous radiation dose on all samples, cells were added to 1 cm polystyrene tubes and filled with PBS. Cells were irradiated in a linear accelerator (KDS Siemens) at room temperature using an electron beam of 18 MeV. Doses between 50 and 500 cGy were applied at a rate of 300 cGy/min. Estimated errors on dose were below 1%. Immediately after radiation cells were cultured as indicated above. HepG2 cells were maintained in culture for 15 days. Cells were counted automatically with a Coulter Multisizer II and verified using a hemocytometer. Cell viability was determined by trypan blue exclusion (0.2%) and propidium iodide labeling.

### 2.2. Determination of total GSH equivalents and synthetic rate of GSH

Molecular forms of GSH equivalents, mainly reduced (GSH) and oxidized form (GSSG), were determined by HPLC as described previously [20]. The dynamic rate of GSH synthesis was determined in

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**Abbreviations:** ARE, antioxidant responsive element; BSO, buthionine-sulfoximine; CAT, chloramphenicol acetyl transferase; DCFDA, 2',7'-dichlorofluorescein diacetate;  $\gamma$ -GCS-HS,  $\gamma$ -glutamylcysteine synthetase heavy subunit; GSH, reduced glutathione; GSSG, oxidized glutathione; Mn-SOD, manganese superoxide dismutase; MRE, metal response element; NF- $\kappa$ B, transcription factor kappa-B; ROS, reactive oxygen species; XRE, xenobiotic response element

cell-free extracts as described in detail previously [21]. Briefly, cell extracts were dialyzed overnight at 4°C to deplete cytosol GSH content to minimize feedback inhibition of GSH on  $\gamma$ -GCS. The GSH synthetic capacity was determined using GSH precursors, glutamate, glycine, cysteine and monochlorobimane as described previously in detail [21]. GSH synthetase activity was assayed using glycine and  $\gamma$ -glutamylcysteine instead of cysteine and glutamate. The rate of GSH formation was monitored as the net rate of fluorescence increase of GSH-mono-chlorobimane adduct catalyzed by GST over time after subtracting the BSO-inhibitable fluorescence signal [21].

### 2.3. Preparation of nuclear extracts and EMSA assay for NF- $\kappa$ B and AP-1

Nuclear extracts were prepared as described previously [22]. Activation of AP-1 and NF- $\kappa$ B was determined by electrophoretic mobility shift assays (EMSA), using consensus probes for AP-1 (5'-CGCTTGATGAGTCAGCCGAA-3') and  $\kappa$ B (5'-AGTTGAGGG-GACTTTCCAGGC-3'), labeled at the 5' end with T4 kinase and  $\gamma$ -<sup>32</sup>P-ATP (3000 Ci/mmol). Proteins (15 mg) were separated through native 6% polyacrylamide gel and visualized by autoradiography.

### 2.4. Analysis of $\gamma$ -GCS-HS mRNA

A cDNA probe for  $\gamma$ -GCS-HS was generated by RT-PCR. An 804-base pair partial cDNA was prepared using rat kidney RNA [23], cloned into pTARGET (Promega, Madison, WI) and sequenced using fmol DNA sequencing system (Promega) to discard PCR-induced mutations. Twenty mg of RNA was size fractionated in a 1% agarose at 2 V/cm under denaturing conditions, and transferred on nylon membrane and fixed with UV. The membranes were prehybridized at 65°C and hybridizations were performed using the <sup>32</sup>P-labeled  $\gamma$ -GCS-HS probe. mRNA levels were calculated relative to the 18S band used as an internal reference control and expressed as percentage of control. Densitometry quantitation of autoradiographs was performed with a densitometer Preference (Seba, France).

### 2.5. Generation of $\gamma$ -GCS-HS promoter and deletion constructs

The  $\gamma$ -GCS-HS promoter was amplified by PCR from genomic

DNA (150 ng) using the following upstream oligonucleotide 5' (+225) GGAGGCGCAGGCAGGAAGACCGA-3' and downstream oligonucleotide 5' (-1088) CAGCCAGACCTTGGGTATTCATG-3' as described previously [11,12]. The resulting promoter fragment (-1088 to +225) was cloned into pTARGET. Using the restriction enzymes *KpnI* and *XhoI*, two fragments of 277 and 1054 bp were subsequently obtained. These fragments and the total promoter (1336 bp) were subcloned into polylinker of the promoterless plasmid pCAT 3 Enhancer vector (Promega). The 1054 bp fragment contained AP-1, AP-2 and MRE regulatory sites, whereas the 277 bp fragment encompassed the NF- $\kappa$ B and ARE sites.

### 2.6. Transient transfection and CAT assay

HepG2 cells were seeded in 6-well plates and cultured until 65–75% cell confluence. pCAT 3 Enhancer and pCAT 3 Control plasmids were transfected using the lipofectamine reagent, according to the manufacturer's instructions. Thirty-six h after transfection, cells were irradiated (400 cGy) as described above. Eight h post-radiation, cell extracts were isolated and protein determined (Bio-Rad, Hercules, CA). Chloramphenicol acetyl transferase (CAT) activity was quantitated by the CAT ELISA (Boehringer Mannheim, Mannheim, Germany).  $\beta$ -Galactosidase expression plasmid (PSVgal, Promega) was cotransfected as an internal control to normalize the transfection efficiency.

### 2.7. Statistical analyses

Statistical analyses for multiple comparisons of mean values between cell preparations were made by one-way ANOVA followed by Fisher's test.

## 3. Results and discussion

The interaction of ionizing radiation with cells in the presence of molecular oxygen leads to generation of ROS, causing oxidative stress. Indeed, an overproduction of ROS stands as one of the major mechanisms involved in the killing of cells by ionizing radiation [24,25]. Since cells may adapt to stress con-

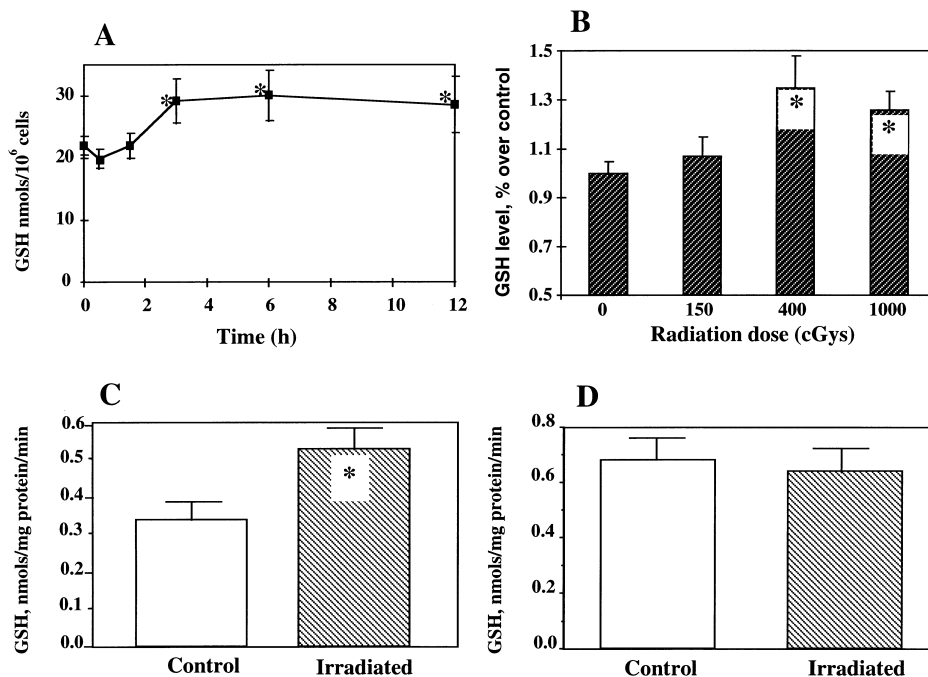


Fig. 1. Regulation of cell GSH and  $\gamma$ -GCS activity by ionizing radiation. A: Cells were radiated (400 cGy) and total cellular GSH equivalents (GSH+GSSG) shown were determined by HPLC. Results were determined in duplicate plates and the mean  $\pm$  S.D. of three different experiments shown. \* $P$  < 0.05 vs. control. B: HepG2 cells were radiated at different doses (0–10 Gy) and GSH content was analyzed by HPLC after 4–5 h post-radiation. Viability of irradiated cells at all doses used was greater than 90%. Results: mean  $\pm$  S.D. of four experiments in duplicate. \* $P$  < 0.05 vs. control. C: Cytosol fraction from control or radiated (400 cGy) cells was isolated (7–8 h) and dialyzed at 4°C to minimize feedback inhibition by GSH. GSH synthetic rate was determined as indicated in Section 2 in the presence of glutamate, glycine and cysteine. D: Activity of GSH synthetase was determined using  $\gamma$ -glutamylcysteine and glycine as GSH precursors and the GSH-bimane fluorescent adduct followed over time. Results are the mean  $\pm$  S.D. of four different experiments. \* $P$  < 0.05 vs. control.

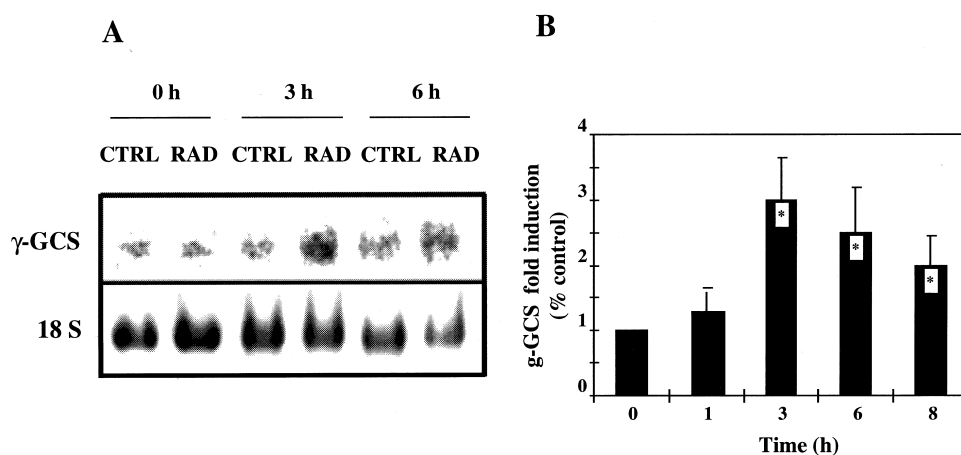


Fig. 2. Regulation of  $\gamma$ -GCS-HS mRNA by ionizing radiation. Total RNA from HepG2 cells was extracted at different times post-radiation and hybridized with cDNA for  $\gamma$ -GCS-HS and analyzed by Northern blot as described in Section 2. Identity of cDNA for  $\gamma$ -GCS-HS was verified by sequencing. 18S band was used as internal control. The variability in the 18S band intensity does not reflect loss of viability of irradiated cells. The magnitude of increase of  $\gamma$ -GCS-HS mRNA was calculated relative to the levels of 18S to minimize the variability in RNA loading between lanes. Percentage increase over control for three different experiments is shown. \* $P < 0.05$  vs. control.

ditions by turning on protective systems to maintain cellular functions under these circumstances, we determined the regulation of GSH in HepG2 cells in response to radiation. We followed the GSH levels in HepG2 cells after exposure to a moderate dose of radiation (400 cGy) (Fig. 1). Within 30 min following radiation, there was a depletion of total GSH equivalents in cells, reflected mainly in the form of reduced GSH ( $17.8 \pm 2.7$  nmol/ $10^6$  cells), that was accompanied by an increase in the form of GSSG ( $2.8 \pm 1.1$  nmol/ $10^6$  cells) compared to control cells ( $22.1 \pm 3.1$  and  $1.4 \pm 0.8$  nmol/ $10^6$  cells for GSH and GSSG, respectively). Such a decrease was transitory as GSH levels recovered to control values by 2 h. Interestingly, however, reduced GSH levels increased above control values ( $23.4 \pm 2.7$  vs.  $31.4 \pm 3.2$  nmol/ $10^6$  cells, for control and irradiated cells, respectively) by 3 h following radiation with minimal effect on GSSG ( $1.8 \pm 0.9$  vs.  $2.1 \pm 1.1$  nmol/ $10^6$  cells), effect that persisted for the next 9–12 h (Fig. 1A). Exposure of cells to lower doses of radiation (100–200 cGy) did not result in significant increase of total GSH levels (Fig. 1B). A similar effect was observed when cells were exposed to a greater radiation dose (1000 cGy), as cellular reduced GSH

levels increased over control values (35–40%) after 5 h post-radiation. The initial depletion of reduced GSH and subsequent decrease in GSH/GSSG, reflecting a radiation-induced oxidative stress, occurred to a greater extent at 1000 cGy than at 400 cGy (4.3 vs. 6.4, GSH to GSSG ratio, respectively), effect that preceded the increment of cellular reduced GSH evoked by radiation. A further indication of the oxidative stress elicited by radiation was the increase in ROS (20–30%), monitored as the fluorescence of cells labeled with DCFDA, a fluorescent probe sensitive to ROS [22]. Despite the evidence of radiation-induced oxidative stress, especially at the dose of 1000 cGy, cells remained intact and viable (>90%), judged by the lack of trypan blue or propidium iodide staining and release of cytosolic enzymes to the extracellular media. These findings indicate that the effects of radiation on GSH homeostasis were not due to the selection of a subpopulation of HepG2 cells that were resistant to radiation.

To assess if the up-regulation of cellular GSH induced by radiation was due to a greater capacity to synthesize GSH, we determined the synthetic rate of GSH in cell-free extracts isolated from control or HepG2 cells after being exposed to

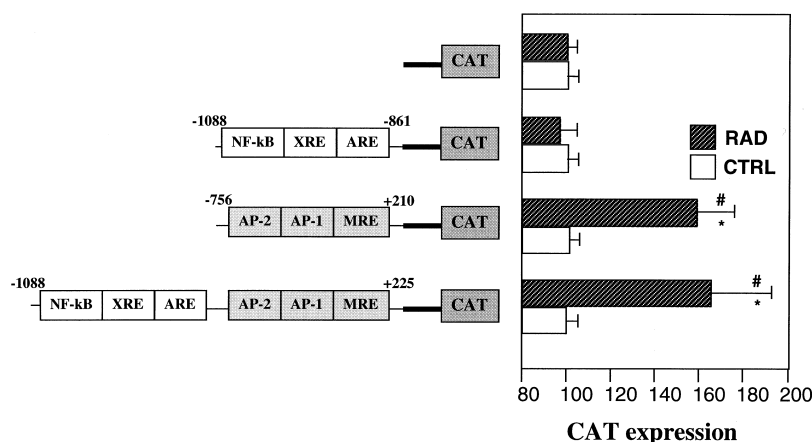


Fig. 3. Effect of radiation on  $\gamma$ -GCS-HS-CAT promoter constructs. Generation of  $\gamma$ -GCS-HS-CAT constructs was accomplished as described in Section 2. After 8 h post-radiation (400 cGy), cell extracts were obtained and used for CAT activity by ELISA normalized for protein content. The pCAT reporter vector did not result in measurable CAT activity in cell extracts. Results are mean  $\pm$  S.D. of  $n=4$  individual experiments. \* $P < 0.05$  vs. control.

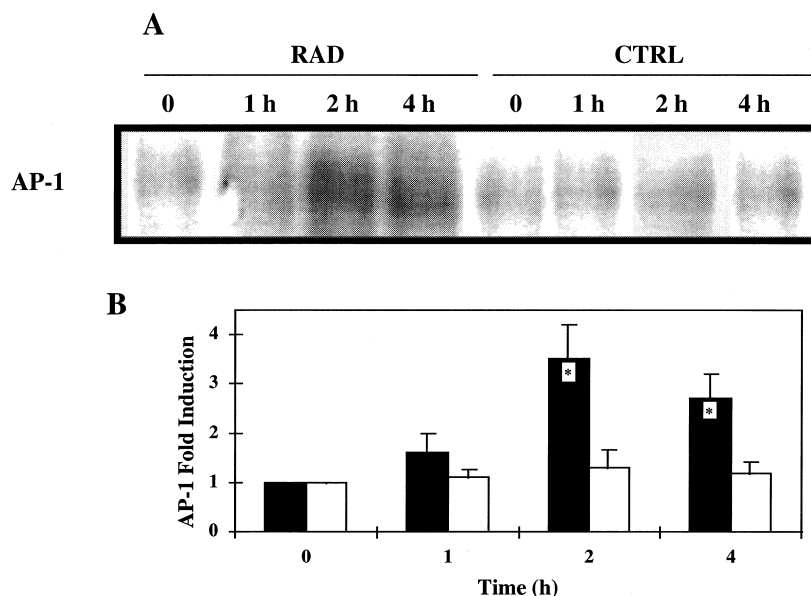


Fig. 4. Activation of AP-1 by radiation. A: Nuclear extracts from irradiated (400 cGy; full bars) and control cells (open bars) were isolated at various times after radiation and incubated in the presence of labeled AP-1 oligonucleotide as described in Section 2. Only retarded band of DNA-AP-1 complex is shown. B: Densitometric quantitation of AP-1 activation. Results are the mean  $\pm$  S.D. of  $n=3$  individual preparations. \* $P < 0.05$  vs. control.

radiation. Cellular extracts were dialyzed to minimize feedback inhibition of GSH on  $\gamma$ -GCS and then incubated with unlimited GSH precursors and cofactors. The dynamic synthetic rate of GSH was determined using monochlorobimane as a probe which forms a highly fluorescent adduct with GSH by a reaction catalyzed by GST transferases [21]. GSH synthesis in cytosol extracts incubated with GSH cofactors (glutamate, glycine and ATP) and cysteine as the sulfur amino acid, reflects the activities of both  $\gamma$ -GCS and GSH synthetase. The GSH synthetic rate increased significantly (40–50%) in cell extracts isolated from HepG2 cells that have been irradiated at 400 cGy compared to control cells (Fig. 1C).  $\gamma$ -Glutamylcysteine, the product of  $\gamma$ -GCS, is the substrate for GSH synthetase to which glycine is added forming the final product, GSH. When  $\gamma$ -glutamylcysteine was used in the *in vitro* synthetic assay instead of cysteine, the formation of GSH reflected the activity of GSH synthetase. In contrast to the results obtained using cysteine as sulfur donor, no change in GSH formation from  $\gamma$ -glutamylcysteine was observed, indicating that radiation did not affect the activity of GSH synthetase (Fig. 1D). Therefore, our findings show for the first time that ionizing radiation confers a greater capacity to synthesize GSH due to an induction in the activity of  $\gamma$ -GCS. Due to the critical role of GSH in determining the survival of cells under stress, our findings suggest that the induction of  $\gamma$ -GCS stands as a vital strategy to control the unwanted consequences of ROS. These revealing findings constitute another example of the induction of protecting enzymes, including  $\gamma$ -GCS, in response to multiple stimuli, such as carcinogens, alkylating drugs, chemotherapeutic agents and cytokines such as IL-6 and TNF [12–17,26–28]. The mechanism whereby unrelated compounds such as chemotherapeutic, alkylating quinones, cytokines and electrophiles lead to an increase in  $\gamma$ -GCS activity is not completely known and may be mediated by the involvement of specific cis-regulatory elements located in the promoter of  $\gamma$ -GCS.

GSH exerts a feedback inhibition on  $\gamma$ -GCS, the regulatory and rate limiting enzyme in GSH biosynthesis [23]. Since a fall in GSH levels preceded its subsequent induction, it is conceivable that a lower feedback inhibition of GSH on  $\gamma$ -GCS may contribute to its induction.  $\gamma$ -GCS is comprised of two subunits, heavy and light chain that are discoordinately synthesized. The catalytic activity resides in the heavy subunit ( $\gamma$ -GCS-HS) whereas the light chain lowers the  $K_m$  for glutamate and increases the feedback inhibition by GSH on the heavy subunit [23]. To assess if the increased  $\gamma$ -GCS activity was paralleled by an up-regulation of  $\gamma$ -GCS-HS mRNA, we determined  $\gamma$ -GCS-HS mRNA levels in HepG2 following radiation. As shown (Fig. 2), Northern blot analysis revealed an increase in the  $\gamma$ -GCS-HS mRNA (2–3-fold) in cells exposed to radiation compared to control, detectable 3 h following radiation.

To determine if the increased  $\gamma$ -GCS-HS mRNA levels induced by radiation reflected a stabilization of their mRNA or were due to a greater transcriptional rate of this gene, we performed functional analyses of a partial  $\gamma$ -GCS-HS promoter fragment subcloned in a CAT reporter vector. As seen in Fig. 3, cells that were transfected with the full length construct –1088/+255, displayed a significant stimulation of CAT activity following radiation. Furthermore, the 5' flanking region of  $\gamma$ -GCS-HS promoter has been recently characterized, identifying several regulatory cis acting elements [11,12,17], including  $\kappa$ B and AP-1 binding sites. NF- $\kappa$ B has been shown to exert a protective role against stress and radiation, perhaps by inducing detoxifying enzymes, such as DT-diaphorase [29,30]. Since radiation leads to activation of NF- $\kappa$ B [31–33] and in view of previous studies that have shown that the transcription of  $\gamma$ -GCS is associated with activation of AP-1 [11,13,34], deletion constructs containing separately AP-1 and NF- $\kappa$ B regions were generated to determine the role of these factors in the transcription of  $\gamma$ -GCS-HS by radiation. Following transient transfection of these constructs into

HepG2 cells, CAT activity was determined in cell extracts isolated from irradiated cells. Compared to stimulation of CAT activity in cell extracts isolated from irradiated HepG2 that were transfected with the full length construct, the shorter construct (277 bp) containing NF- $\kappa$ B-like and XRE/ARE-like responsive elements, resulted in minimal CAT expression by radiation. High levels of transcription activation of  $\gamma$ -GCS-HS promoter were induced by radiation when cells were transfected with the deletion plasmid  $-756/+216$  containing AP-1/MRE cis-regulatory elements, as the magnitude of CAT activity induced by radiation was similar to that seen with the full length plasmid (Fig. 3).

To establish whether radiation induced DNA binding to the AP-1 binding site in the  $\gamma$ -GCS promoter, we performed electrophoretic mobility shift assays with nuclear extracts prepared from irradiated HepG2 cells using a radiolabeled oligonucleotide encompassing the AP-1. As seen in Fig. 4, there was a time dependent increase in AP-1 activation which was maximal after 4 h post-radiation. The specificity of binding for AP-1 was established from competition of labeled AP-1 oligonucleotide with a molar excess of unlabeled AP-1 (not shown). Altogether, these results indicate that NF- $\kappa$ B is not required in the regulation of  $\gamma$ -GCS-HS by radiation, whereas involvement of other putative enhancer elements including AP-1 are required for such an effect. At present we cannot completely discard the involvement of other cis-regulatory elements located further upstream in the 5' flanking region of  $\gamma$ -GCS-HS gene, which have been recently described [17]. Our finding indicating a role for AP-1 in the transcription of  $\gamma$ -GCS is in agreement with previous reports [11,13,34]. The mechanism underlying the activation of AP-1 by radiation has not been fully characterized. In this regard, it has been shown that radiation results in the release of ceramide, a lipid signalling intermediate, from the plasma membrane of irradiated cells, by hydrolysis of sphingomyelin. It is conceivable that ceramide may play a role in the activation of AP-1 by radiation, as has been shown previously for other agonists which use ceramide as a signal transducer, through activation of stress-activated protein kinases [35–38]. In addition, since ceramide causes oxidative stress by generating ROS [39,40], these reactive species may also play a role in the activation of AP-1.

Despite our findings showing that the up-regulation of the catalytic subunit of  $\gamma$ -GCS is sufficient to raise the levels of cellular GSH, the effect of radiation on the regulatory subunit of  $\gamma$ -GCS (the light subunit) remains to be determined. Although previous studies have indicated a minimal change in the regulatory subunit of  $\gamma$ -GCS [16,17], recent findings have shown a coordinate induction of both heavy and light subunits of  $\gamma$ -GCS in response to redox cycling quinones [41].

Finally, it can be envisioned that cells challenged by oxidative stress induced by radiation may up-regulate the GSH levels to cope with the potentially deleterious effects of free radicals. Indeed, our findings indicate that exposure of HepG2 cells to moderate doses of radiation up-regulates the  $\gamma$ -GCS-HS mRNA levels and activity leading to greater content of the radioprotective antioxidant, GSH. Although the magnitude of the increased levels of GSH in irradiated cells may appear modest, the capacity to replenish cell GSH rather than the absolute levels of GSH would constitute an efficient tool to confer protection against the overproduction of ROS and the subsequent oxidative stress generated by radiation.

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